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Endocrine responses to repeated adrenocorticotropic hormone administration in free-ranging elephant

Molly McCormley
University of the Pacific, mmccorml88@gmail.com

ENDOCRINE RESPONSES TO REPEATED ADRENOCORTICOTROPIC HORMONE ADMINISTRATION IN FREE-RANGING ELEPHANT SEALS

by

Molly McCormley

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APPROVED BY:

Thesis Advisor: Jane Khudyakov, Ph.D.

Thesis Co-Advisor: Cory Champagne, Ph.D.

Committee Member: Dan Crocker, Ph.D.

Committee Member: Zachary Stahlschmidt, Ph.D.

Department Chair: Craig Vierra, Ph.D.

Dean of Graduate School: Thomas H Naehr, Ph.D.

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DEDICATION

This thesis is dedicated to friends and family who always supported and believed in me. Especially to my mom and dad who were there when I needed to cry hysterically because there were no places in Stockton that sold women's Carrhartts. Also to my amazing sister, who I feel like should be the older sister, because I look up to her so much, thanks for being there for me when I needed you.

Also to my amazing friends, who supported me and understood that I would come out my hole at some point. Thank you for being there for me when I called and for letting me vent (which I'm sure were the same things over and over again) nonstop. I really have the best friends in the world.

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Endocrine responses to repeated adrenocorticotropic hormone administration in free-ranging elephant seals

Abstract

by Molly McCormley

University of the Pacific
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Understanding the physiological response of marine mammals to anthropogenic stressors can inform marine ecosystem conservation strategies. Stress stimulates release of glucocorticoid (GC) hormones, which increase energy substrate availability while suppressing energy-intensive processes. Exposure to repeated stressors can potentially affect an animal's ability to respond to and recover from subsequent challenges. To assess the endocrine response of a marine mammal to repeated stressors, we administered adrenocorticotropic hormone (ACTH) to free-ranging juvenile northern elephant seals (*Mirounga angustirostris*; n=7) once daily for four days. ACTH administration induced significant, but transient (<24 h) elevation in circulating cortisol levels ($p < 0.0001$). These increases did not vary in magnitude between the first ACTH challenge on day 1 and the last challenge on day 4. In contrast, aldosterone levels

remained elevated above baseline for at least 24 hours after each ACTH injection ($p < 0.001$), and responses were greater on day 4 than day 1 ($p < 0.01$). Total triiodothyronine (tT3) levels were decreased on day 4 relative to day 1 ($p < 0.01$), while reverse triiodothyronine (rT3) concentrations increased relative to baseline on days 1 and 4 ($p < 0.001$) in response to ACTH, indicating a suppression of thyroid hormone secretion. There was no effect of ACTH on the sex steroid dehydroepiandrosterone (DHEA). These results suggest that elephant seals are able to mount adrenal responses to multiple ACTH challenges. However, repeated stress results in facilitation of aldosterone secretion and suppression of tT3, which may impact osmoregulation and metabolism. We propose that aldosterone and tT3 are informative additional indicators of repeated stress in marine mammals.

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Chapter 1: Introduction

Stress due to natural and anthropogenic disturbance can greatly affect wildlife populations (1). Chronic stress, which can be caused by sustained or repeated challenges, can lead to homeostatic overload, impairing an animal's ability to respond appropriately to additional stressors and potentially impacting survival (2, 3). Changes in the health and abundance of wildlife populations, especially of top predators like marine mammals, can have ecosystem-wide consequences (4). Marine mammals routinely experience physiological challenges such as hypoxia and prolonged fasting, and amphibious species (e.g. pinnipeds, or seals and sea lions) are exposed to both terrestrial and aquatic stressors (5, 6). Increased anthropogenic activity in coastal and marine habitats, such as ecotourism, noise pollution, commercial fishing, and human-driven habitat loss, in addition to natural environmental challenges (e.g. prey availability), can have cumulative impacts on marine species (4). Such impacts have been correlated with modern declines in marine mammal populations (1, 4, 7). Recent research on stress in marine mammals has focused on animals' responses to acute stressors (e.g. a single endocrine response; 8, 9-12). However, little information exists on impacts and

indicators of chronic stress (e.g. endocrine response to repeated or sustained stressors) in free-ranging marine mammals and most other wildlife species (13, 14). Therefore, evaluation of the physiological impacts of both acute and chronic stress in marine mammals is needed to understand how populations may respond to anthropogenic and environmental disturbance over time and can help inform conservation management strategies (15-17).

The Stress Response

The mammalian stress response is mediated by catecholamines and the hypothalamic-pituitary-adrenal (HPA) axis (18, 19). Activation of the HPA axis by an acute stressor induces a series of cascading events, culminating in the release of glucocorticoids (GC), mineralocorticoids, and androgens from the adrenal gland as a result of stimulation by corticotropin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) from the hypothalamus and pituitary gland, respectively (18).

Glucocorticoids (e.g. cortisol) are considered the primary stress hormones; they exert their effects by binding to intracellular glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) in target tissues and influencing gene expression (18). Produced in the zona fasciculata cells of the adrenal cortex, GCs exert numerous physiological effects that include increased catabolism of metabolic stores required to meet immediate energetic needs and suppression of energetically demanding functions (e.g. reproduction and immune responses; 20, 21, 22). Negative feedback at the receptor level is responsible for regulating

responses to GCs to avoid potentially deleterious long-term effects, such as depletion of energy reserves needed for maintenance and reproduction. Depletion of energy stores can have detrimental consequences for marine mammal species that fast such as during molting and breeding. While many studies of stress in wild animals have concentrated solely on GC measurements (23-26), no consensus GC profile that characterizes sustained or repeated stress responses has been determined (13). Therefore, a suite of additional hormones should be measured to fully evaluate the true physiological burden and endocrine characteristics of repeated stress (27). These include other adrenal hormones (aldosterone and dehydroepiandrosterone, DHEA) and components of the hypothalamic-pituitary thyroid (HPT) axis.

The mineralocorticoid aldosterone, produced in the zona glomerulosa cells of the adrenal cortex, induces osmoregulatory actions, and it had not been studied in the context of the stress response in terrestrial mammals until recently (28). In most mammals, aldosterone research had focused mainly on its role within the renin-angiotensin-aldosterone system (RAAS), regulated by changes in renal pressure. Within the RAAS, aldosterone is released in response to a decrease in blood pressure and function in reabsorption of salt and water by the kidney, causing an overall increase in renal and arterial pressure (28). In marine mammals, a number of studies have demonstrated significant increases in aldosterone secretion in response to perturbation (8, 9, 11, 29, 30), suggesting that mineralocorticoids may be an especially important component of the stress

response in mammals adapted to hypersaline environments, with potential osmoregulatory or cardiovascular costs (6, 28, 31-33).

The sex steroid precursor DHEA (commonly measured in its more abundant sulfated form, DHEA-S) is produced by the zona reticularis cells of the adrenal cortex in response to HPA axis activation in vertebrates (34-37). Prolonged upregulation of cortisol synthesis during repeated stress may influence the synthesis and release of DHEA, which has been shown to counteract glucocorticoid activity and alter lipid metabolism, insulin sensitivity, and adipocytokine production in adipose tissue (38, 39). Dysregulation of DHEA during repeated stress may thus impact reproduction and metabolism.

HPA axis activation also directly affects the HPT axis by decreasing thyroid-stimulating hormone release and inhibiting biologically active triiodothyronine (T3) synthesis, leading to an increase in the non-biologically active reverse triiodothyronine (rT3; 40, 41). Thyroid hormones, which regulate basal metabolic rate, thermogenesis, and lipid metabolism, have also been shown to be important for fasting metabolism in elephant seals (42, 43). Suppression of the thyroid axis by repeated stress would have significant detrimental consequences on the ability of marine mammals to effectively undertake key life history stages that require fasting, such as molting and breeding.

Elephant Seals

Northern elephant seals (*Mirounga angustirotris*) regularly haul out (come out on land), for periods of up to several months, fasting the entire time, during

which they undergo breeding and lactation, or molting in the winter or spring, respectively (44). Juveniles haul out during an additional period during the fall. Due to this temporal predictability and relative ease of research handling, considerable amounts of data are available on baseline physiological functions in elephant seals, including natural variability in GCs, thyroid, and other hormones (42, 43, 45, 46). In particular, previous research in juveniles has shown that natural fluctuations of corticosteroid levels in response to life history phases, such as molting and breeding, are not associated with the fall haul-out period (43, 47). Furthermore, sedation procedures have been developed in this species that permit experimental manipulation and sample collection with minimal handling artifact (8). Therefore, the elephant seal is an ideal free-ranging study system for experimental manipulation in marine mammals, without the confounding of psychological stress, and they can ultimately serve as a proxy for more endangered or less accessible species.

Objective

The objective of this study was to characterize and compare the endocrine responses to acute and repeated stress in a well-studied marine mammal, the northern elephant seal. Hormone secretion patterns may change in response to one or multiple periods of stress, resulting in facilitation (an increase in response over time) or attenuation (a decrease in response over time) (3). We used the hormone responses to a single and multiple stress challenges to evaluate the potential for discrimination between acute and repeated stress states. We simulated acute and repeated stress with daily administration of synthetic ACTH for four consecutive

days and compared responses to the first (initial) and to fourth (repeated) administration.

Chapter 2: Methods

Experimental Design

The stress manipulation experiment was conducted during Aug–Oct 2016 at Año Nuevo State Park, San Mateo County, CA, USA. Experiments were conducted using seven juvenile (approximately 0.8 year-old) northern elephant seals. ACTH was administered once daily for four consecutive days to simulate an acute and repeated stress response. Serial blood samples were collected pre-ACTH (0-hour) and post-ACTH (1-8 hours) ondays 1 and 4 to assess the effects of ACTH administration on cortisol, aldosterone, and thyroid hormones (Fig. 1).

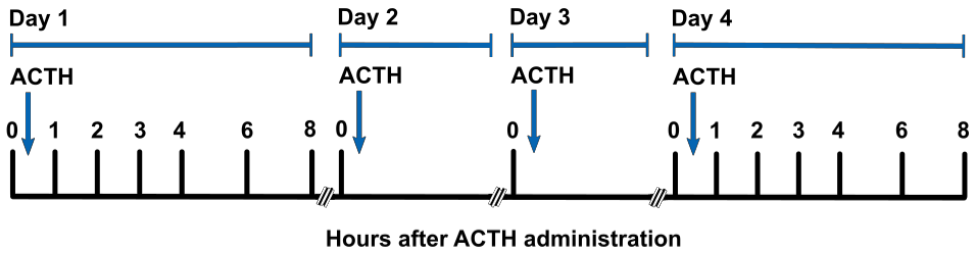

Figure 1. Overview of the study design, timing of ACTH administration, and blood sample collection during the 4-day experiment. ACTH was administered once daily. Blood samples were collected immediately prior to each ACTH administration (0-hour samples) each day. Post-ACTH samples were collected on days 1 and 4 at 1, 2, 3, 4, 6, and 8 hours following the administration, as indicated above.

Study Subjects

All animal handling procedures were conducted under National Marine Fisheries Service permit 19108 and approved by Sonoma State University and University of the Pacific Institutional Animal Care and Use Committees and Department of the Navy Bureau of Medicine and Surgery. Juvenile northern elephant seals regularly haul out during fall and are reliably found on successive days at the rookery. Natural fluctuations of corticosteroid levels in response to life history phases, such as molting and breeding, are not associated with this haul out period (43, 47). The study subjects, two females and five males, were selected based on size and good body condition (Table 1). All animals were healthy individuals with normal body sizes for their age (99-137 kg) which permitted similar mass-specific doses of ACTH (Table 1). Seals were weighed by suspension from a tripod and scale (MSI tension dynamometer, Seattle, WA, USA), and marked with rear flipper tags (Dalton, Oxon, UK) for identification. All study animals resumed normal activity and remained at the rookery after the experiment was completed on each day.

Table 1. Sex, mass, and mass-specific ACTH dose (U/kg) for each subject are shown. ACTH, adrenocorticotropic hormone; SD, standard deviation.

Subject	Sex	Mass	ACTH (U/kg)
1	M	125	0.16
2	M	119	0.19
3	M	130	0.15
4	M	125	0.16
5	M	137	0.15
6	F	103	0.19
7	F	99	0.20
Mean (SD)		**120 (13)**	**0.17 (0.02)**

Sedation and ACTH Administration

Seals were chemically immobilized using an intramuscular injection of ~1 mg/kg tiletamine–zolazepam HCl (Telazol, Fort Dodge Animal Health, Fort Dodge, IA, USA), and sedation was maintained with intravenous doses of ketamine and diazepam (Fort Dodge Animal Health, Fort Dodge, IA, USA) as needed to complete baseline sample collection and ACTH administration. This anesthetic procedure does not affect GC concentrations (48). On days 1-4, the 0-hour samples were obtained from the extradural vein using an 18 G, 3.25-inch spinal needle within 18.0 ± 5.5 minutes of initial sedation. Samples were drawn directly into chilled vacuum collection tubes (serum, heparinized, and EDTA-treated vacutainers; BD Franklin Lakes, NJ, USA).

Twenty units (U; mean mass-specific dose: 0.17 ± 0.02 U/kg) of a synthetic ACTH preparation (11, 29); Wedgewood Pharmacy, Swedesboro, NJ, USA) were administered via intramuscular injection into the posterior flank of

each animal following immobilization once daily for 4 days, 24.0 ± 0.7 hours apart (Fig. 1). The same injection sites were used, alternating from the left to the right side each day. On days 2 and 3, animals were allowed to recover from anesthesia immediately after ACTH administration and no response samples were taken. On days 1 and 4, samples were collected for 8 hours after ACTH administration as described below.

Post-ACTH Sampling

On days 1 and 4, an indwelling catheter (16 G×20 cm, MILA International Inc, Florence, KY, USA) was inserted into the extradural vessel and attached to a 60-inch extension tube (MILA International Inc, Florence, KY, USA) after ACTH administration, following which the animals were allowed to recover from anesthesia. Additional doses of ketamine and diazepam were administered prior to blubber tissue biopsy sampling for a related study on days 1 and 4 as described previously (11). Serial blood samples (1, 2, 3, 4, 6, and 8 hours after ACTH administration) were collected via extension tubing while the animals rested in the field. Blood samples were drawn into syringes and immediately transferred to chilled (on ice) vacuum collection tubes.

Sample Storage

Blood samples were stored chilled (on ice) in a field cooler for 1 – 3 hours until processing. Serum and plasma were isolated by centrifugation at 3,000 x *g*

for 15 min, kept frozen on dry ice until return to the laboratory, and stored at -80°C until further analysis.

Hormone Analyses

Serum cortisol was measured in duplicate using a radioimmunoassay (RIA; MP Biomedicals, Burlingame, CA, USA) previously validated for northern elephant seals (11). Aldosterone was measured in all plasma samples in triplicate using an enzyme-linked immunosorbent assay (ELISA; 11-ALDHU-E01, lot# 161410; Alpco, Salem, NH, USA). The aldosterone ELISA was validated by demonstrating parallelism of diluted samples to the standard curve (Fig. 2), and complete spike recovery as described below.

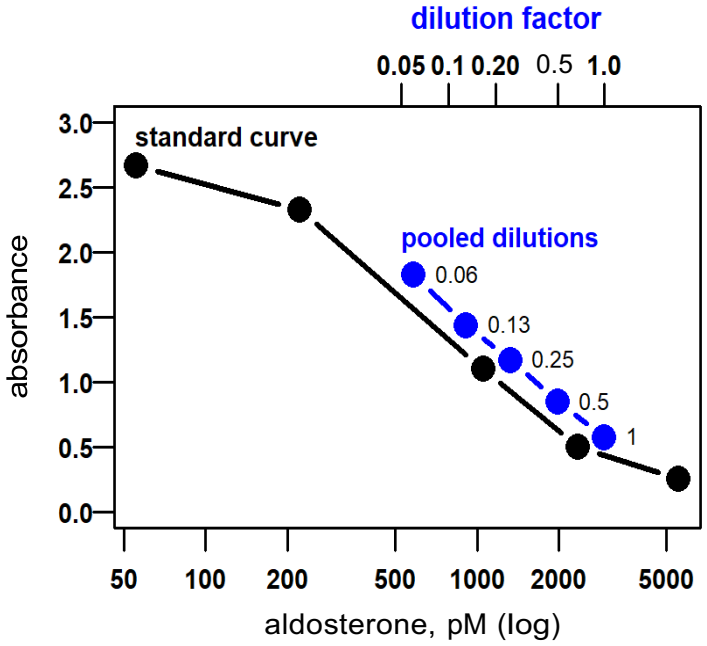

Figure 2. The enzyme-linked immunosorbent assay (ELISA) used to measure aldosterone was validated in part by showing parallelism of serially diluted pooled seal serum with the standard curve.

Matrix interference effects were tested by adding varying volumes of pooled seal serum (pooled from 6 samples, added 1- 20 µL) to a known volume of kit standard (30 µL); total aldosterone concentrations was corrected for added standard (49). There was no association between the corrected aldosterone concentration and volume of elephant seal serum added ($p=0.11$). The calculated hormone recovery was 104 ± 6%. Average intra-assay coefficients of variation (CV) between sample replicates were 2.6% for cortisol and 5.9% for aldosterone.

Thyroid hormones (tT3, rT3) and DHEA-S were measured in a subset of serum samples (0-hour samples from days 1–4; 4-hour and 8-hour samples from

days 1 and 4) in duplicate using RIAs (MP Biomedicals, Burlingame CA). Total T3 and reverse T3 RIAs were previously validated for northern elephant seals (9). DHEA-S was validated by demonstrating parallelism of diluted samples to the standard curve. Matrix interference effects were validated by adding varying volumes of pooled seal serum to a known volume of kit standard, like our previous aldosterone validation; total DHEA-S concentrations were corrected for added standard (49). The calculated hormone recovery was 97 ± 5%. Average intra-assay coefficients of variation (CV) between sample replicates were 3.2% for tT3, 3.5% for rT3, and 2.3% for DHEA-S.

Statistical Analyses

Statistical analyses were conducted using RStudio statistical software version 1.0.136 (50). Linear mixed models (LMMs), with subject ID as a random effect, were used to explore hormone variation among samples repeatedly collected from individuals after ACTH administration. Degrees of freedom were estimated using the Kenward-Rogers approximation and p-values determined using the lmerTest package (51); post-hoc comparisons were performed using the multcomp package (52).

Responses of each hormone to ACTH administration were assessed with LMMs within days 1 and 4; if significant differences were detected, we followed with a Dunnett's post-hoc test against the 0-hour sample from that day. We evaluated hormone recovery from ACTH administration using only the pre-

ACTH (0-hour) samples from each day; if differences were detected, we again followed with a Dunnett's test against the day 1 0-hour sample.

We explored associations between hormones using LMMs. Goodness of fit was calculated with marginal r-squared (mR^2) statistics for mixed models (53) employed in the MuMIn package (54).

We compared the hormone responses to initial and repeated ACTH administrations (days 1 and 4, respectively) in three ways. (1) For hormones measured in the full set of samples (cortisol and aldosterone), we calculated the total hormone response to ACTH administration on day 1 and day 4 by summing the hormone vs time polygons relative to their initial concentrations and normalized to an eight-hour sample duration (see Fig. 3) to calculate an integrated area under the curve (AUC) value for each subject. These AUC values for days 1 and 4 were then compared using paired t-tests. We assessed the change in hormone concentration (Δ) as the change in concentration from the 0-hour sample to the peak for that day (0-hour subtracted from peak). These values were then compared between days 1 and 4 using paired t-tests to determine changes in magnitude of response for hormones measured in a subset of samples (e.g. tT3). (2) The maximal (peak) response of each hormone to ACTH was also compared between days 1 and 4 using a paired t-test. (3) Hormones that were measured in a subset of samples (tT3, rT3, and DHEA-S; each measured in pre-ACTH (0-hour) and 4- and 8-hour post ACTH samples) were compared using paired t-tests between respective sample times (4-hour and 8-hour samples on days 1 and 4). Removal of outliers (e.g. seal 5) did not have an effect on statistical significance,

and data from this animal were retained in the analyses. Due to limited sample size, the effects of sex were not determined within this analysis.

Chapter 3: Results

ACTH administration caused a significant increase in cortisol concentrations relative to 0-hour samples on days 1 and 4 (Dcortisol = 1144 nM on day 1 and 2047 nM on day 4; LMM: $F_{6, 36} = 20.7$ and 10.1 for days 1 and 4, respectively, $p < 0.0001$ for both days). Cortisol levels were significantly elevated in all post-ACTH relative to pre-ACTH samples on days 1 and 4 (Dunnett's test, $p < 0.05$; Fig. 3A).

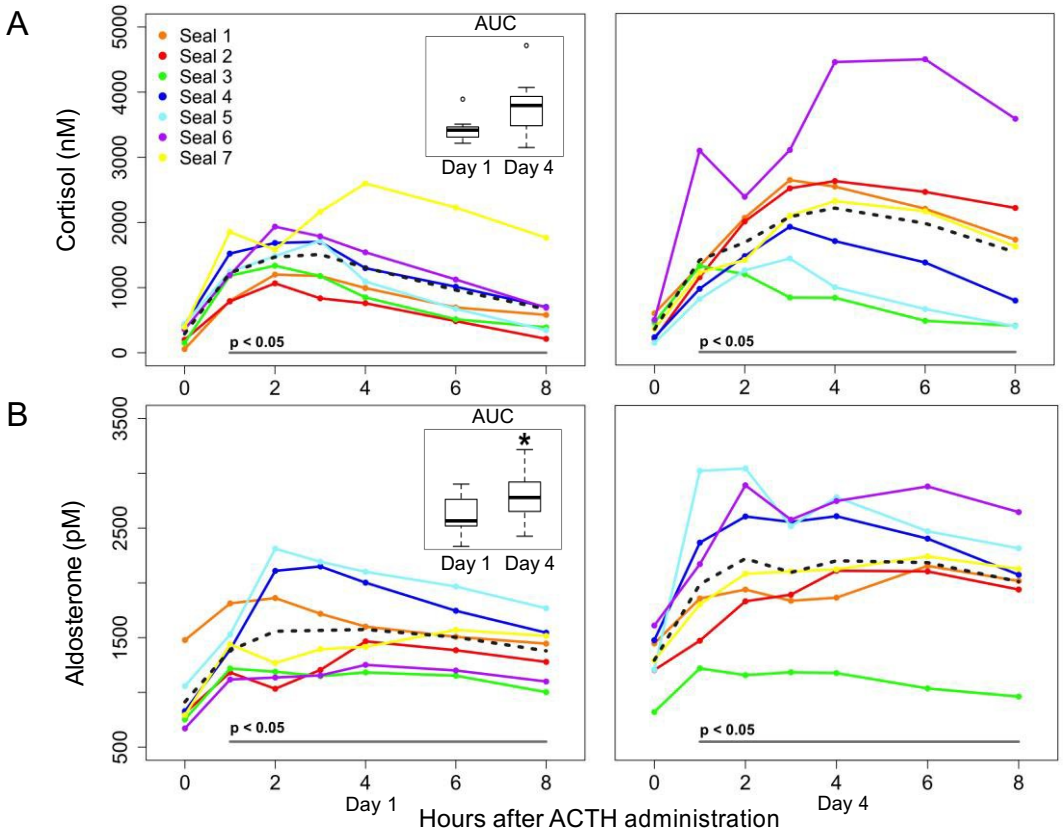

Figure 3. (A) Cortisol concentrations (1-8 h) increased relative to pre-ACTH (0-hour) levels following ACTH administration on days 1 (left panel) and 4 (right panel; LMM: $F_{6, 36} = 20.7$ and 10.1 for days 1 and 4, respectively, $p < 0.0001$). Total cortisol response (area under the curve, AUC, inset plot) and peak cortisol levels did not differ between days 1 and 4 (paired t-test, AUC: $p = 0.12$, peak: $p = 0.13$). (B) Aldosterone increased relative to pre-ACTH (0-hour) concentrations following ACTH administration on days 1 (left panel) and 4 (right panel; LMM: $F_{6, 36} = 9.76$ and 13.6 for days 1 and 4, respectively, $p < 0.0001$). Aldosterone concentrations were significantly elevated on day 4 relative to day 1 (AUC, inset plot; paired t-test, $p < 0.05$). The dashed lines show mean hormone levels. * denotes statistical difference in AUC concentrations between days. The gray bar at the bottom of each figure denotes significant differences in post-ACTH hormone concentrations relative to 0-hour levels on each day.

Cortisol levels in pre-ACTH samples did not vary among days 1–4 (LMM, p = 0.24), indicating that cortisol recovered within 24 hours of each ACTH administration (Fig. 4A).

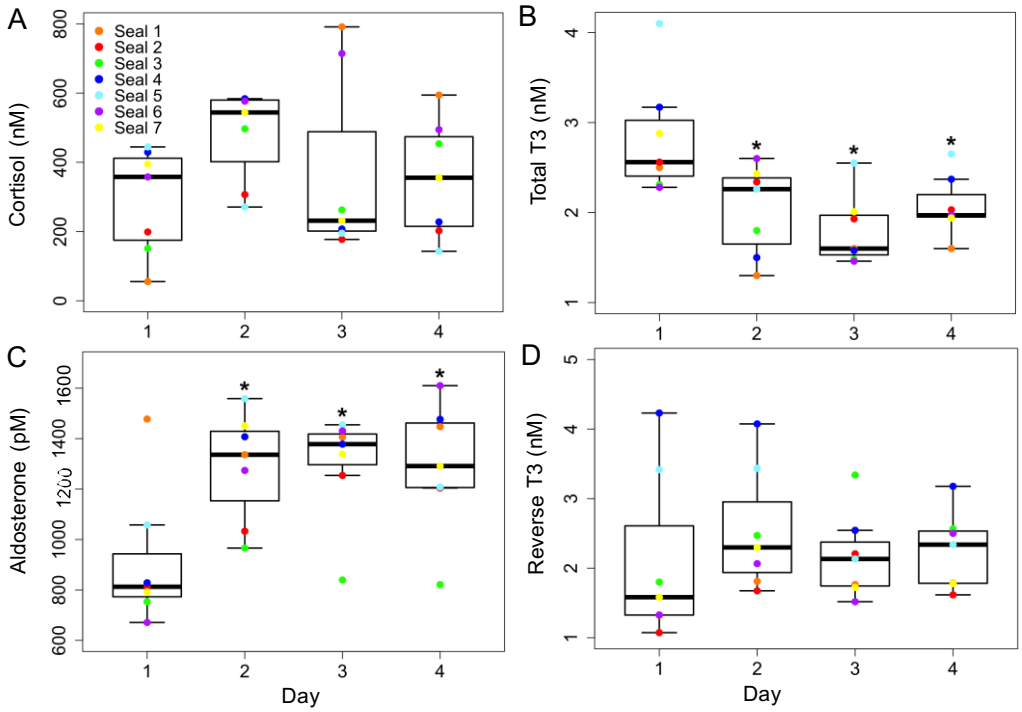

Figure 4. Hormone measurements in samples collected prior to ACTH administration (0-hour) each day of the experiment. 0-hour cortisol (A), and reverse T3 (D) levels recovered to baseline within 24 hours after each ACTH administration. Aldosterone (C) 0-hour levels on days 2-4 were elevated relative to baseline (day 1 0-hour) concentrations (LMM: $F_{3, 18} = 8.06$, $p < 0.001$). Total T3 (B) 0-hour concentrations on days 2-4 were suppressed relative to day 1 0-hour concentrations (LMM: $F_{3, 18} = 10.5$, $p < 0.0001$). * denote significant differences between pre-ACTH hormone levels on day 1 and subsequent days. Whiskers are at most 1.5 standard deviations above and below the 3rd and 1st quartile respectively.

There was high variability in the animals' individual cortisol responses to repeated ACTH administration (Table 2).

Table 2. Serum cortisol and aldosterone concentrations measured on days 1 and 4 of the repeated stress experiment in seven juvenile elephant seals. Baseline samples (Base) were taken before ACTH administration. Peak is the highest concentration measured after ACTH administration within the 8-hour sampling period. * denotes statistical difference in peak and baseline mean concentrations between days 1 and 4. Cort, Cortisol; Aldo, Aldosterone; SD, standard deviation.

	Day 1				Day 4			
Subject	**Base Cort**	**Peak Cort**	**Base Aldo**	**Peak Aldo**	**Base Cort**	**Peak Cort**	**Baseline Aldo**	**Peak Aldo**
1	55	1199	1478	1861	593	2641	1447	2155
2	199	1066	813	1466	202	2626	1204	2111
3	151	1337	753	1189	454	1323	821	1184
4	429	1703	828	2149	228	1924	1477	2609
5	444	1719	1058	2312	143	1436	1208	3044
6	358	1936	671	1251	494	4497	1610	2891
7	394	2599	792	1570	356	2319	1291	2242
Mean (SD)	**290 (142)**	**1651 (483)**	**913 (255)**	**1685 (403)**	**359 (156)**	**2395 (988)**	**1294* (234)**	**2319* (573)**

Some seals exhibited facilitation (n=4), or an increase in the magnitude of cortisol secretion in response to the fourth ACTH injection relative to the first (e.g. seal 6: Dcortisol = 1578 nM and 4003 nM on days 1 and 4, respectively). Other animals displayed attenuation, or a decrease in magnitude of the cortisol response to the fourth ACTH injection relative to the first (n=2; e.g. seal 3: Dcortisol = 1185 nM and 870 nM on days 1 and 4, respectively). One animal showed little difference between cortisol responses to ACTH on day 1 and day 4 (seal 5: Dcortisol = 1275 nM and 1294 nM on days 1 and 4, respectively). Peak cortisol concentrations and total cortisol secretion in response to ACTH were not

significantly different between days 1 and 4 (paired t-test, peak: $p = 0.13$, AUC: $p = 0.12$; Table 2, Fig. 3A).

Aldosterone levels were significantly elevated in all post-ACTH samples relative to pre-ACTH samples on days 1 and 4 (Dunnett's test, $p < 0.0001$, Fig. 3B). Aldosterone concentrations in 0-hour samples from days 2–4 were significantly elevated relative to the day 1 0-hour sample (LMM: $F_{3, 18} = 8.06$, $p < 0.001$; Dunnett's test against day 1 0-h sample, $p < 0.001$; Fig. 4C). Peak concentrations and total secretion of aldosterone in response to ACTH were significantly higher on day 4 than on day 1 (paired t-test, $p < 0.01$ and $p < 0.05$, respectively; Table 1, Fig. 3B). This suggests that aldosterone levels did not recover within 24 hours of each ACTH dose and that the aldosterone response was sensitized by multiple ACTH administrations.

Thyroid hormone (tT3, rT3) and DHEA-S concentrations were measured in 0-hour samples from days 1–4 and in samples collected 4 and 8 hours after ACTH administration on days 1 and 4. ACTH caused a significant decrease in tT3 levels on both days 1 and 4 (Fig. 5A; LMM: $F_{2, 12} = 25.3$, 18.4 for days 1 and 4, respectively, $p < 0.0001$). On day 1, tT3 concentrations were significantly decreased in the 8-hour sample (Dunnett's test, $p < 0.001$), but not 4-hour sample ($p = 0.44$) relative to pre-ACTH levels. On day 4, tT3 was significantly decreased related to pre-ACTH levels in both in both 4-hour and 8-hour post-ACTH samples (Dunnett's test, $p < 0.001$). The magnitude of tT3 responses to ACTH were not different between days 1 and 4 (paired t-test, $p = 0.08$). Total T3 levels in 0-hour samples from days 2–4 were significantly lower than the pre-ACTH 0-hour

sample from day 1 (LMM: $F_{3, 18} = 10.5$, $p < .0001$; Fig 4B), and were further suppressed in post-ACTH samples (4 and 8-hours) from day 4 relative to those from day 1 (paired t-test, $p < 0.001$).

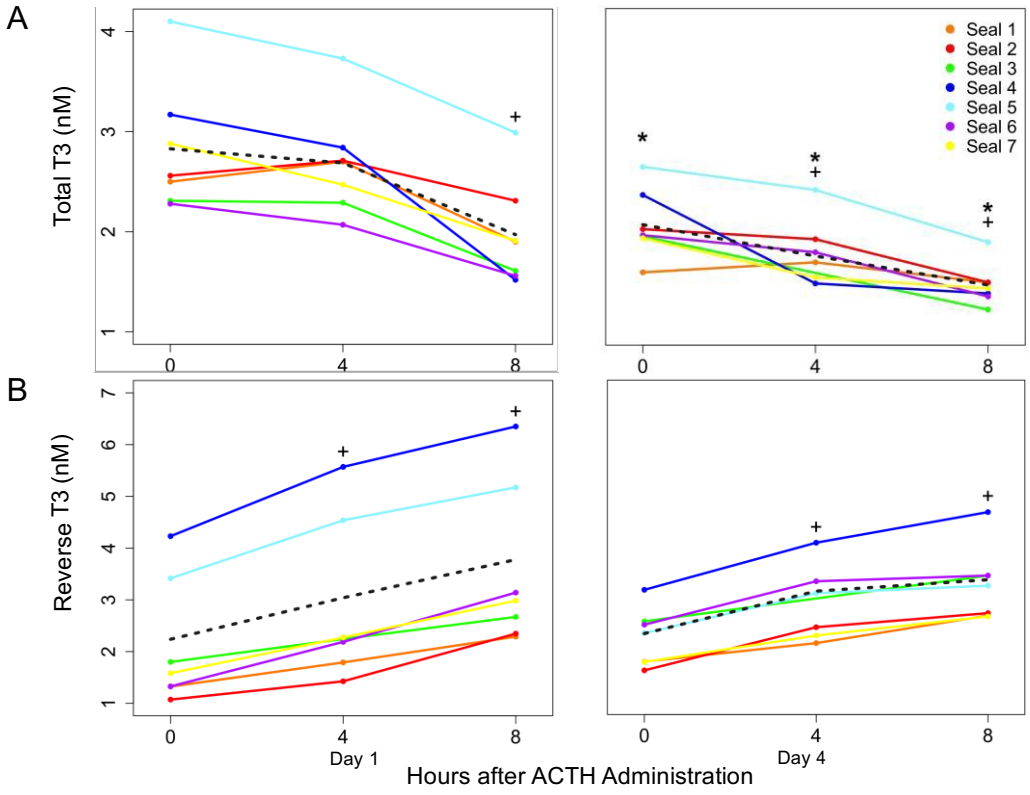

Figure 5. Total T3 (A) concentrations measured 8 hours after ACTH administration were significantly decreased relative to pre-ACTH (0-hour) levels on days 1 (left panel). Total T3 concentrations measured 4 and 8 hours post-ACTH were significantly decreased relative to pre-ACTH (0-hour) levels on day 4 (right panel; LMM: $F_{2, 12} = 25.3$, 18.4 for days 1 and 4, respectively, $p < 0.0001$). Total T3 concentrations were suppressed on day 4 relative to day 1 (paired t-test, $p < 0.001$). Reverse T3 (B) concentrations 4 and 8 hours after ACTH administration were significantly increased relative to pre-ACTH (0-hour) levels (LMM: $F_{2, 12} = 57.0$ and 18.4 for days 1 and 4, respectively, $p < 0.0001$), but did not differ between days. The dashed lines show mean hormone concentrations. + denotes hormone values that were significantly different ($p < 0.05$) from the 0-hour sample on that day. * denotes values that were significantly different (paired t-test, $p < 0.001$) between days 1 and 4.

Reverse T3 levels were significantly increased 4 and 8 hours after ACTH administration relative to 0-hour concentrations on days 1 and 4 (Fig. 5B; LMM: $F_{2, 12} = 57.0$ and 18.4 for days 1 and 4, respectively, $p < 0.0001$; Dunnett's test against the 0-hour for each day) sample, $p < 0.001$; paired t-test, $p < 0.05$). However, rT3 concentrations did not vary between 0-hour samples on days 1–4 (LMM, $p = 0.95$; Fig. 4D). There were no significant differences in post-ACTH rT3 concentrations on days 1 and 4 (paired t-test, $p = 0.76$ and $p = 0.50$ for days 1 and 4, respectively). DHEA-S concentrations were not affected by ACTH (Fig. 6; LMM, $p = 0.06$) and did not differ between days 1– 4 (paired t-test, $p = 0.31$).

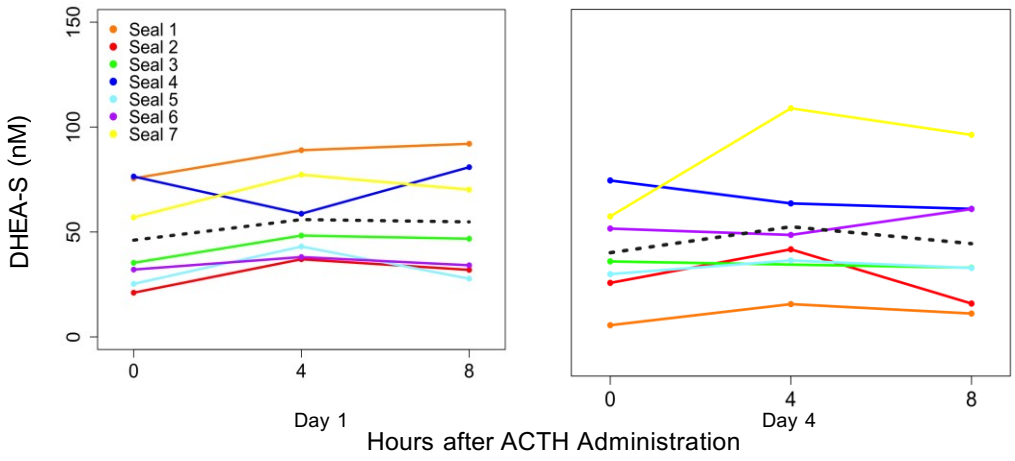

Figure 6. DHEA-S concentrations did not change following ACTH administrations on day 1 (left panel; $p > 0.05$) or day 4 (right panel; $p > 0.05$). The dashed lines show mean hormone concentrations.

Cortisol was positively associated with aldosterone (LMM: $F_{1, 107} = 105$, $p < 0.0001$; $mR^2 = 0.38$; Fig. 7A) and rT3 (LMM: $F_{1, 49} = 12.0$, $p < 0.001$; $mR^2 = 0.09$; Fig. 7C). Aldosterone was negatively associated with tT3 (LMM: $F_{1, 54} =$

8.26, $p < 0.001$; $mR^2 = 0.12$; Fig. 7B**)** and positively associated with rT3 (LMM: $F_{1, 50} = 5.88$, $p < 0.001$; $mR^2 = 0.10$; Fig. 7D).

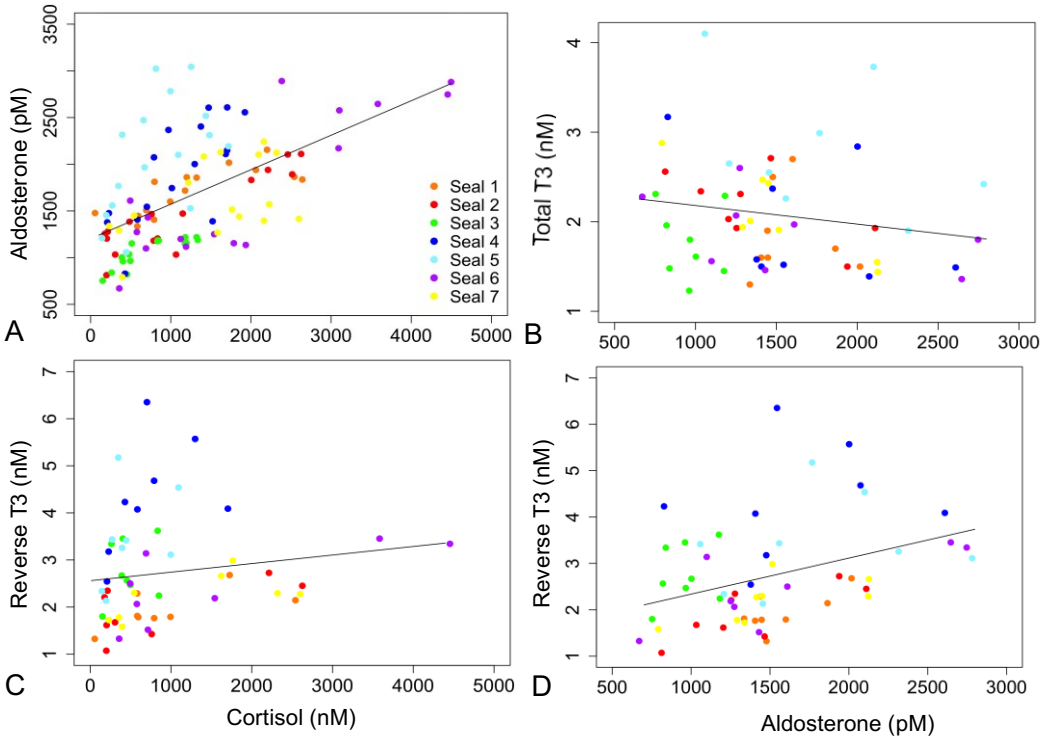

Figure 7. Associations between hormone levels in samples collected during the experiment. Cortisol and aldosterone (A) were positively associated (LMM: $F_{1, 106.8} = 105$, $p < 0.0001$; $mR^2 = 0.38$). Aldosterone and total T3 (B) were negatively associated (LMM: $F_{1, 53.5} = 8.26$, $p < 0.001$; $mR^2 = 0.12$). Cortisol and reverse T3 (C) were positively associated (LMM: $F_{1, 7.47} = 6.38$, $p < 0.05$; $mR^2 = 0.29$). Aldosterone and reverse T3 (D) were positively associated (LMM: $F_{1, 7.66} = 5.88$, $p < 0.05$; $mR^2 = 0.26$).

Chapter 4: Discussion

We evaluated the endocrine responses of free-ranging marine mammals to repeated HPA axis activation. We administered ACTH once daily for four consecutive days to juvenile northern elephant seals to simulate the physiological responses that a marine mammal may experience due to frequently occurring natural or anthropogenic stressors. Elephant seals showed similar magnitude of cortisol release on day 4 as on day 1, suggesting that they maintained adrenal sensitivity to repeated ACTH stimulation for at least 4 days. Aldosterone and tT3, however, showed different responses to the initial and repeated ACTH administration: while the magnitude of aldosterone response was increased, the tT3 response was diminished. These differential responses to single and repeated stressors suggests that these hormones may be useful additional markers to aid in distinguishing between acute and repeated stress states.

Cortisol significantly increased in response to ACTH on days 1 and day 4 of the study. While cortisol release was much higher than has been previously reported for other marine mammals in response to acute exogenous ACTH (e.g. harbor seals and steller sea lions, 10, 12), values were within range of GC concentrations previously reported in juvenile northern elephant seals (8, 11, 29), except in one case in which a subject's day 4 peak cortisol concentration (4497 nM) was about 1.6 -fold higher than what was previously described (29). However, the stress response samples were only collected within 2 hours after

ACTH administration in the aforementioned study, whereas they were collected for 8 hours in this experiment.

There was no difference detected between the day 1 and day 4 cortisol responses as AUC and peak values did not differ between the two days. All subjects had elevated cortisol concentrations for 7 hours following ACTH administration on days 1 and 4. Although response samples were not taken on days 2 and 3 during this study, pilot trials have shown that similar responses are expected on days 2 and 3 (data not shown). This likely resulted in a substantial stress load that may approach a chronically stressed state. Despite this, all animals retained a high level of adrenal responsiveness to ACTH and cortisol levels returned to baseline within 24 hours of each ACTH dose in all seven study animals, suggesting that juvenile northern elephant seals retain the capacity to mount four repeated stress responses.

The wide variation in responses among individual subjects, however, suggests that physiological differences between specific animals account for the variable cortisol response to repeated stressors. While sex differences may account for some of the variation, as the highest responses on day 1 and 4 were in females (seal 7 and seal 6 respectively), these two females showed similar cortisol response concentrations to the males on the other day. Variation in individual GC responses to exogenous ACTH or handling has been observed in other marine mammals (e.g. Steller sea lions, 12), in fish, and in terrestrial species (55-57), which suggests that GC concentrations are highly variable across species and stressors and may therefore be less reliable quantifiable indicators of specific stress states (13).

Aldosterone significantly responded to ACTH administration both on day 1 and day 4. While aldosterone levels were found to be slightly higher than those reported in harbor seals in response to ACTH (10), they were within the range of previously reported values for juvenile northern elephant seals (8, 11). Repeated ACTH administration significantly affected aldosterone secretion, as peak values and AUC values after the fourth ACTH administration (day 4) were higher relative to peak values and AUC values measured after the first administration (day 1). All subjects, had elevated aldosterone concentrations for at least 7 hours (and likely longer) following ACTH administration on day 1 and 4, and remained elevated across all four days.

All seals, with the exception of one, exhibited facilitation of aldosterone secretion on day 4 compared to day 1, including individuals that displayed atteniation or no change in their cortisol responses (Fig. 2). The single outlier animal had similar aldosterone and cortisol secretion patterns on days 1 and 4. Unlike cortisol, aldosterone concentrations did not recover to baseline values 24 hours after the first ACTH administration and remained elevated in the pre-ACTH (0-hour) samples on days 2, 3, and 4. This suggests that aldosterone is sensitive to repeated HPA axis activation, which may impair negative feedback. Therefore, aldosterone may be an informative indicator of repeated stress states in marine mammals.

A significant increase in aldosterone levels in response to acute stressors (e.g. ACTH administration, cold stress, veterinary examination) has been observed in multiple marine mammal species, including pinnipeds such as

northern elephant seals (8, 9) and Pacific harbor seals (10, 30), and in cetaceans, such as Atlantic bottlenose dolphins (58-60) and spotted dolphins (61). The ability to regulate aldosterone concentrations in the contexts of normal and stress physiology may be especially essential for marine mammals that ingest large amounts of salt and water during foraging and feeding, and must maintain appropriate osmoregulatory function while diving (19, 62, 63). In terrestrial animals, exposure to psychosocial and cold stress has been shown to directly increase renin production, which is likely mediated by the sympathetic nervous system (64-66). Angiotensin II has also been shown to increase in response to stress in rats, directly increasing aldosterone levels by activating type 1 angiotensin II receptors in the adrenal cortex (67). Therefore, it is possible that aldosterone secretion in response to stress in marine mammals occurs indirectly, via stress-induced sympathetic activation of RAAS (58). However, the positive correlation between cortisol and aldosterone concentrations and direct responsiveness to ACTH reported in this and other marine mammal studies suggest that the HPA axis may play a major role in aldosterone regulation in response to stress (8).

ACTH administration had no effect on DHEA in this study. This was unexpected as significant increases in DHEA have been reported in primates and cattle in response to repeated stress, suggesting that this hormone precursor may be used to buffer effects of sustained elevation of GCs (68, 69). However, experiments using white-crowned sparrows have shown that DHEA is either

unaffected or suppressed in response to acute stress. Thus, some vertebrates may not rely on its anti-glucocorticoid effects (70).

While ACTH administration had a significant suppressive effect on tT3 on day 1 and day 4, ACTH significantly increased rT3 concentrations on both days. Total T3 values reported in this study were in some cases almost two-fold higher than tT3 values described for adult male northern elephant seals, even after suppression by ACTH (9). Reverse T3 values were almost 50-fold greater than measured in adult males, but within the range of values previously reported in juvenile northern elephant seals in response to exogenous ACTH (8, 9). Total T3 levels decreased within 8 hours of the first ACTH administration and remained suppressed relative to the day 1 0-hour baseline sample for the remainder of the experiment. However, while the first ACTH dose significantly decreased tT3 production and subsequent doses caused further suppression, the magnitude of tT3 suppression on days 1 and 4 was not different. These results are not unexpected as cortisol has been shown to suppress conversion of T4 to the biologically active T3 and promote its conversion to the biologically inactive rT3 via its effects on deiodinase enzymes (71, 72). Accordingly, levels of rT3 increased within 4 hours of ACTH administration, which is consistent with other studies in this species (Champagne et al., 2015). However, unlike tT3, there was no difference between rT3 response to the first and fourth ACTH challenge. This may be due to differential regulation of deiodinase enzymes (73, 74) or liver clearance rates, as rT3 is unbound by carrier proteins and therefore may be cleared more rapidly than tT3 (75). However, rT3 and cortisol levels were

positively associated in response to a single ACTH challenge in juveniles and adult male elephant seals sampled early in the breeding season (8, 9). This suggests that thyroid sensitivity to HPA axis activation is dependent on the number of ACTH challenges (single vs. repeated) and life-history stage in elephant seals, and that repeated HPA axis activation has a significant inhibitory effect on thyroid hormone production.

Associations between the HPA and HPT axes have been shown in laboratory rodents (76) and other marine mammal species including belugas, in which capture and handling stress also resulted in suppression of tT3 (77). The inhibitory effect of stress on tT3 has significant implications as thyroid hormones regulate basal metabolic rate via their effects on mitochondrial proliferation and lipid and protein metabolism (19, 78). Stress-induced disruption in thyroid hormone production can therefore greatly impact health and fitness in species that fast during critical life history stages (e.g. breeding, molting, migration). For example, mean thyroid hormone levels do not change in male northern elephant seals over their extended breeding fast, enabling them to maintain elevated metabolic rates for its duration. Furthermore, the ability of individual seals to elevate T3 is associated with higher daily energy expenditure and breeding success (42, 79). Metabolic suppression, even for short time periods during this life history stage, could lead to an inability to sustain reproductive behavior, decreasing fitness.

This study demonstrates the importance of measuring multiple endocrine variables in addition to GCs in response to repeated ACTH challenges. Cortisol

responses to HPA axis stimulation varied between individuals but not by the number of stress challenges. GC responses to stress are therefore likely to be influenced by physiological variability between animals more significantly than aldosterone and tT3, which displayed consistent differences in responses to single and multiple ACTH challenges. Sensitization of the aldosterone response and suppression of tT3 by repeated ACTH administration suggests mechanisms by which repeated stress may affect marine mammal health and fitness (i.e. altering osmoregulation or cardiovascular adjustments and suppressing metabolism). These data suggest that aldosterone and thyroid hormone levels may be informative biomarkers of repeated stress in marine mammals. Determining varying baseline concentrations will help further contextualize HPA facilitation or attenuation in elephant seals and other marine mammals (43). Characterizing the further consequences of stress, including alterations in gene expression and metabolism, may also offer added insight into the differing responses to acute and repeated stress. Our study demonstrated endocrine responses to simulated repeated stressors such as those that would be elicited by repeated anthropogenic disturbance in marine mammals.

Anthropogenic activity is likely to continue to increase in marine ecosystems, increasing the likelihood of human interactions with marine life that elicit stress and compound the physiological extremes already experienced by these animals as a consequence of their marine environment (4). Geographical ranges of many marine mammals, including threatened species, overlap with areas associated with high human activity like fishing, shipping, or military sonar use

(80-82). Therefore, the ability to accurately measure homeostatic loads in marine mammals exposed to anthropogenic disturbance and predict their effects on fitness is increasingly important for conservation practitioners working to protect marine ecosystems and their top predators (14)

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